Expression of collagen and related growth factors in rat tendon and skeletal muscle in response to specific contraction types

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Acute exercise induces collagen synthesis in both tendon and muscle, indicating an adaptive response in the connective tissue of the muscle–tendon unit. However, the mechanisms of this adaptation, potentially involving collagen-inducing growth factors (such as transforming growth factor-β-1 (TGF-β-1)), as well as enzymes related to collagen processing, are not clear. Furthermore, possible differential effects of specific contraction types on collagen regulation have not been investigated. Female Sprague–Dawley rats were subjected to 4 days of concentric, eccentric or isometric training ($n = 7$–9 per group) of the medial gastrocnemius, by stimulation of the sciatic nerve. RNA was extracted from medial gastrocnemius and Achilles tendon tissue 24 h after the last training bout, and mRNA levels for collagens I and III, TGF-β-1, connective tissue growth factor (CTGF), lysyl oxidase (LOX), metalloproteinases (MMP-2 and -9) and their inhibitors (TIMP-1 and 2) were measured by Northern blotting and/or real-time PCR. In tendon, expression of TGF-β-1 and collagens I and III (but not CTGF) increased in response to all types of training. Similarly, enzymes/factors involved in collagen processing were induced in tendon, especially LOX (up to 37-fold), which could indicate a loading-induced increase in cross-linking of tendon collagen. In skeletal muscle, a similar regulation of gene expression was observed, but in contrast to the tendon response, the effect of eccentric training was significantly greater than the effect of concentric training on the expression of several transcripts. In conclusion, the study supports an involvement of TGF-β-1 in loading-induced collagen synthesis in the muscle–tendon unit and importantly, it indicates that muscle tissue is more sensitive than tendon to the specific mechanical stimulus.

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Tendon and the extracellular matrix (ECM) of skeletal muscle are collagen-rich tissues that are important for muscle function, especially in relation to force transmission. The fibrillar collagens, type I and III, are the major components of tendon and muscle ECM. In tendon, type I collagen is predominant, while both types are expressed at equal levels in muscle ECM (Light & Champion, 1984). In cases of training leading to muscle hypertrophy and increased strength, it seems imperative that these collagen-rich tissues adapt in order to sustain the integrity of the muscle–tendon unit (Kjaer *et al.* 2006). *In vivo* human studies indicate that collagen synthesis increases in both muscle and tendon in response to acute exercise (Langberg *et al.* 1999; Heinemeier *et al.* 2003; Moore *et al.* 2005; Miller *et al.* 2005), and, similarly, prolonged training appears to elevate type I collagen synthesis in human tendon (Langberg *et al.* 2001). These findings indicate that collagen synthesis, and possibly collagen mRNA expression, in tendon and muscle is regulated by exercise/training, and a few studies have in fact shown elevated expression levels of type I and III collagen mRNA in both muscle and tendon in response to loading in animals (Han *et al.* 1999; Archambault *et al.* 2001; Koskinen *et al.* 2001; Olesen *et al.* 2006*b*). Meanwhile, the knowledge on how specific contraction types (e.g. concentric *versus* eccentric) may influence collagen expression and synthesis in a differential manner is very limited. In the present study we have measured

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changes in tendon and muscle expression of type I and III collagen in response to short-term training involving either pure, concentric, eccentric or isometric contractions in rats.

It is acknowledged that loading induces collagen synthesis in muscle and tendon, but the mechanisms responsible for mediating this effect are still unclear. Several observations point to transforming growth factor-β-1 (TGF-β-1) as an essential mediator of mechanically induced collagen synthesis in a variety of cell types (Gutierrez & Perr, 1999; Nakatani *et al.* 2002; Kim *et al.* 2002; Lindahl *et al.* 2002), including patella tendon fibroblasts (Yang *et al.* 2004), and this growth factor may well act as a link between loading and collagen synthesis in the muscle–tendon unit. In addition to TGF-β-1, connective tissue growth factor (CTGF), a member of the CCN (CTGF, cyr61 and Nov) family, could be implicated in this process, as it is involved in mechanically induced collagen synthesis (Hishikawa *et al.* 2001; Schild & Trueb, 2002), possibly acting down-stream of TGF-β-1 (Grotendorst *et al.* 1996; Duncan *et al.* 1999).

Besides induction of collagen synthesis, mechanical loading of tendon and muscle tissue has been suggested to induce certain changes in the collagen structure, possibly involving increased collagen cross-linking (Kovanen *et al.* 1984; Kubo *et al.* 2002) but also degradation (Langberg *et al.* 2001; Olesen *et al.* 2006*a*). Therefore, training may change the expression of enzymes involved in collagen processing, such as lysyl oxidase (LOX), which facilitates cross-linking of collagen (Kagan & Li, 2003), and matrix metalloproteinases (MMPs) and their inhibitors, which regulate degradation of collagen molecules (Visse & Nagase, 2003).

The aim of the present study was to investigate the specific effects of concentric, eccentric and isometric short-term training in rats, with regard to the expression of type I and III collagen mRNA, in tendon and muscle tissue. Furthermore, to identify possible mediators of loading-induced collagen regulation in these tissues, we studied the changes in expression of collagen-inducing growth factors as well as enzymes related to collagen processing and turnover. Our earlier findings indicate that an anabolic response is induced in tendon tissue by short-term training and that this response is independent of the contraction type performed by the muscle (Heinemeier *et al.* 2007). Based on this, we hypothesize that a loading-induced expression of collagen and collagen-related growth factors and enzymes in tendon will not depend on contraction type. In muscle tissue, however, there are several indications that the anabolic effect is more pronounced after eccentric loading compared with other loading types (Hather *et al.* 1991; Hortobagyi *et al.* 2000; Farthing & Chilibeck, 2003; Heinemeier *et al.* 2007) and the regulation of muscle connective-tissue-related genes may well follow a similar pattern.

Methods

Training of rats

The model used for training of rats in the present study has been described earlier (Adams *et al.* 2004; Heinemeier *et al.* 2007).

Animals. Young adult female Sprague–Dawley rats, weighing 238 ± 2 g (mean $\pm$ S.E.M.), were assigned randomly to three groups (minimum of 7 in each group). Each group was subjected to involuntary concentric training (CON), eccentric training (ECC), or isometric training (ISO). Rats were housed in groups in standard vivarium cages on a 12 : 12 h light–dark cycle and had *ad libitum* access to standard rat chow and water. The study was conducted according to the American Physiological Society Guiding Principles in the Care and Use of Animals, and the protocol was approved by the University of California Institutional Animal Care and Use Committee.

Electrical muscle stimulation. Prior to each training bout, the rats were lightly anaesthetized with an intraperitoneal injection of ketamine (30 mg kg^{-1}), xylazine (4 mg kg^{-1}) and acepromazine (1 mg kg^{-1}). To confirm a satisfactory level of anaesthesia, the animals were carefully monitored during the training sessions with pinch tests on the feet for reflex behaviour. Stainless steel wire electrodes, coated with Teflon, were used for stimulation. The electrodes were introduced subcutaneously in the region adjacent to the poplateal fossa, via 22-gauge hypodermic needles (which were withdrawn leaving the electrode in place). Before insertion, a section of the Teflon was removed, leaving the wire exposed in the area lateral and medial to the sciatic nerve and allowing for field stimulation of the nerve. The electrodes were attached to the output poles of a Grass stimulus isolation unit interfaced with a Grass S8 stimulator. This allowed for delivery of current to the sciatic nerve and thus muscle activation.

When stimulation electrodes were in place, the rats were positioned in a specially designed training platform previously described (Caiozzo *et al.* 1992). The left leg was positioned in a footplate, which was attached to the shaft of a Cambridge model H ergometer. The voltage and stimulation frequency ($\sim$50 Hz) were adjusted to produce maximal isometric tension.

Training. During all training bouts the sciatic nerve of the left leg was stimulated for 2 s with 18 s between stimulations. Sets consisted of 10 stimulations and were separated by 5 min rest. Rats were trained for four consecutive days with two sets on day 1, three sets on day 2, and four sets on day 3 and 4. After each training session the electrodes were removed. The right leg served as control.

The training protocols were controlled by computer via a digital-to-analog board (model DDA-06, Keithley

Table 1. Primers

MRNA	Sense primer	Anti-sense primer
COL1A1[a]	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG
COL3A1[a]	TGATGGGATCCAATGAGGGAGA	GAGTCTCATGGCCTTGCGTGTTT
TGF-β-1[a]	CCCCTGGAAAGGGCTCAACAC	TCCAACCCAGGTCCTTCCTAAAGTC
TGF-β-1[b]	GCTGCTGACCCCCACTGATA	CCAACCCAGGTCCTTCCTAA
CTGF[a]	CAGGCTGGAGAAGCAGAGTCGT	CTGGTGCAGCCAGAAAGCTCAA
CTGF[b]	GGCGAGTCCTTCCAAAGCAGTT	GGTCTTAGAACAGGCGCTCCAC
LOX[a]	CAGGCACCGACCTGGATATGG	CGTACGTGGATGCCTGGATGTAGT
MMP-2[a]	CTGGGTTTACCCCCTGATGTCC	AACCGGGGTCCATTTTCTTCTTT
MMP-9[a]	GGATGTTTTTGATGCCATTGCTG	CCACGTGCGGGCAATAAGAAAG
TIMP-1[a]	ATAGTGCTGGCTGTGGGGTGTG	TGATCGCTCTGGTAGCCCTTCTC
TIMP-2[a]	GGACACGCTTAGCATCACCCAGA	GTCCATCCAGAGGCACTCATCC
GAPDH[a]	CCATTCTTCCACCTTTGATGCT	TGTTGCTGTAGCCATATTCATTGT
RPLP0[a]	AGGGTCCTGGCTTTGTCTGTGG	AGCTGCAGGAGCAGCAGTGG

[a]Primers for real-time RT-PCR. [b]Primers for Northern probe generation.

Instruments) used to control footplate movement and to trigger the stimulus. A separate analog-to-digital board (DAS-16) was used to acquire force measurements (100 Hz acquisition). Data acquisition, control of stimulus triggering and footplate movements were programmed by using LabTech Note-book (Laboratory Technologies). Data analysis was conducted by using Acqknowledge software (Biopac Systems). Force output was monitored in real time on the computer screen during each contraction.

Rats of the ISO group had their right foot placed in the footplate at an angle of ∼44 deg relative to the tibia. The footplate angle was fixed during muscle stimulation. For the CON group, the ergometer allowed the footplate to move from 44 deg to 64 deg after the development of maximal isometric tension in the beginning of muscle contraction. For the ECC group, the footplate was moved from 44 deg to 24 deg after the development of maximal isometric tension. The movement rate was limited to 29 deg s^{-1} in the CON and ECC groups in order to maintain force development. It is important to note that muscles trained under these conditions generate forces that are significantly different among the three training modes, i.e. eccentric trained muscles generated the most force, while the concentric trained muscle generated the least amount of force (see Results).

Tissue sampling and storage

Twenty-four hours after the last training bout the rats were killed via an intraperitoneal injection of Pentosol euthanasia solution (Medical-Pharmex) at a dose of 0.4 ml kg^{-1}. The medial gastrocnemius including the Achilles tendon, from both legs, was dissected free and the mid-section of the muscle belly and the Achilles tendon were cut out and stored at −80°C for later use.

RNA extraction. Total RNA from all tissue types was extracted according to the method described by Chomczynski & Sacchi (1987). Muscle tissue was homogenized in TRI-reagent (MRC) with a Polytron homogeniser (Ultra-Turrax T8, Ika labourtechnik, Staufen, Germany), while tendon tissue was homogenized in TRI-reagent using a bead-mixer (Retsch, MM300) with the aid of 3 mm steel beads. The tubes containing tendon tissue, TRI-reagent and beads, were shaken at 24 Hz for 60 s and then cooled on ice. This was repeated seven times.

Following homogenization, BCP (1-bromo-3-chloropropane) (MRC) was added (100 μl per 1000 μl TRI-reagent) in order to separate the samples into an aqueous and an organic phase. In tendon samples, glycogen was added (120 μg per 1000 μl TRI-reagent) to improve RNA precipitation. Following isolation of the aqueous phase, RNA was precipitated using isopropanol. The RNA pellet was then washed in ethanol and subsequently dissolved in RNase-free water. All samples were weighed prior to RNA extraction. RNA concentrations were determined by spectroscopy at 260 nm. To account for absorbance of the glycogen added to the tendon samples, mock extractions were made in which no tissue was present, and the mean absorbance of these negative controls ($n = 3$) was subtracted from all test values. A good RNA quality was ensured by gel electrophoresis.

Real time PCR

Total RNA (500 ng from muscle and 200 ng from tendon) was converted into cDNA in 20 μl using the OmniScript reverse transcriptase (Qiagen, CA, USA) according to the manufacture's protocol. For each target mRNA, 0.25 μl cDNA was amplified in a 25 μl SYBR Green PCR reaction containing 1 × Quantitect SYBR Green Master Mix (Qiagen, CA, USA) and 100 nM of each primer (Table 1).

The amplification was monitored real-time using the MX3000P real-time PCR machine (Stratagene, CA, USA). The threshold cycle (C_t) values were

related to a standard curve made with the cloned PCR products and specificity was confirmed by melting curve analysis after amplification. The general range of C_t values was 15–30. The large ribosomal protein P0 (RPLP0) had been chosen as internal control, assuming RPLP0 mRNA to be constitutively expressed (Dheda *et al.* 2004). To validate this assumption another unrelated 'constitutive' RNA, GAPDH mRNA, was measured and RPLP0 was normalized to GAPDH. However, the RPLP0/GAPDH ratio was not stable and we chose to normalize mRNA data to the weight of the tissue needed for extracting the amount of RNA used for cDNA synthesis (500 ng RNA for muscle and 200 ng RNA for tendon). This choice of normalization has been discussed in our earlier publication where other data from the present study were presented (Heinemeier *et al.* 2007).

Northern blotting

Probe preparation. Human cDNA was amplified by PCR, using primers specific for the target genes (Table 1). The purified PCR products were ligated into the *Sma*I site of the pBlueScript II SK(+) vector and competent DH5α *E. coli* cells (Invitrogen, Carlsbad, CA, USA) were transformed with the plasmids. Single positive colonies were isolated, and cell cultures were grown and then purified with the High Pure Plasmid Isolation kit (Roche). Size and orientation of the inserted PCR products were tested by asymmetric restriction digest. Using the purified plasmids as templates, the inserts were amplified by PCR with 5′ biotinlylated M13 primers and non-biotinylated M13 primers complementary to the sequences flanking the inserts. The resulting PCR products were separated into single strands and the biotinylated strands were isolated with the use of streptavidin dynabeads. Radioactive probes complementary to the biotinylated DNA strands, were generated with the aid of Exonuklease-free Klenow DNA polymerase (StripEZ, Ambion) and isolated for use in the hybridization procedure.

Blotting and hybridization. Samples containing 460 ng of RNA for muscle, and 200 ng for tendon, were mixed with formaldehyde loading buffer (Ambion) and run on a formaldehyde agarose gel. The gel was dyed in SYBR Green II (Cambrex) and scanned on a fluorescence scanner (Imager FX, Biorad) in order to verify the integrity of the RNA. Gels were then blotted onto Appligene nylon membrane (Qbiogene, Irvine, CA, USA) by alkaline capillary transfer. Membranes were hybridized overnight at 50°C with 10 million counts min^{-1} per blot of probe in 6 ml hybridization buffer. After removal of excess probe, the blots were exposed on a phosphorscreen (Biorad).

Quantification. Phosphoscreen images were visualized using the phosphoimager (Imager FX, Biorad) and band intensity was measured with the use of Quantity One software (Biorad). All values were normalized to a reference mRNA (pooled samples) that was run on the gels at intervals alongside the test samples. 28S ribosomal RNA was intended for further normalization. However, as tissue weight was used for normalization of real-time RT-PCR data (discussed above), the same procedure was followed for the Northern data to allow comparison between the two methods.

Number of samples in each group

For muscle, all samples were successfully analysed (with both real-time RT-PCR and Northern blotting) and all groups included seven to nine samples: for tendon, four, six and eight samples for control, CON, ECC and ISO groups, respectively, and six, seven and nine samples for trained CON, ECC and ISO groups, respectively, were successfully analysed with real-time RT-PCR. Northern analysis was performed successfully on six to eight samples for all tendon groups.

Statistics

All mRNA data (except MMP-9 mRNA values) were log-transformed before statistical analyses and are presented as geometric means ± back-transformed S.E.M.

A two-way ANOVA on contraction × training was performed using the SAS (Statistical Analysis System) procedure mixed with autoregressive modelling. If the two-way ANOVA was significant, individual differences between trained and untrained leg within each contraction type, and between contraction types, were tested with a *post hoc* test (Bonferroni). Otherwise, training effect was tested against control on all contraction types combined. For MMP-9 mRNA, which was expressed at very low levels, a χ^2 test was performed to test if the number of samples containing detectable levels of MMP-9 mRNA was altered as a result of the training intervention.

Differences were considered significant when $P < 0.05$.

Results

Force–time integral

The force–time integral was significantly different between all three contraction modes ($P < 0.01$), with the greatest force–time integral being obtained during eccentric loading and the lowest during concentric loading (Fig. 1).

Type I collagen

Achilles tendon levels of collagen type I, α1 (COL1A1) mRNA increased 5- to 6-fold in response to all types of training and no difference was seen between training types ($P > 0.05$) (Fig. 2*A*). In gastrocnemius muscle, only

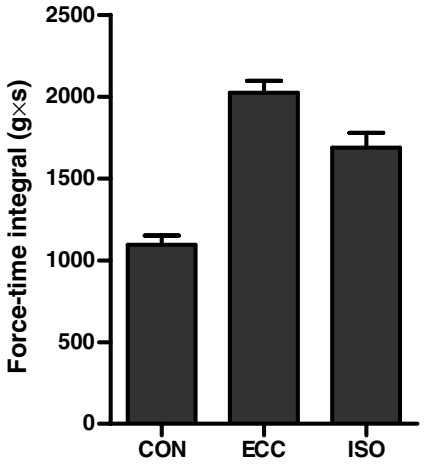


Figure 1. Mean force–time integral over the 2 s stimulation period for CON, ECC and ISO contractions
There was a significant difference in force–time integral between all 3 contraction types ($P < 0.01$).

eccentric and isometric training increased COL1A1 mRNA expression ($P < 0.05$), and eccentric training tended to have a greater effect than concentric ($P = 0.1$) (Fig. 2*B*).

Type III collagen

In tendon, collagen type III, $\alpha 1$ (COL3A1) mRNA increased markedly (15- to 20-fold) in response to all types of training ($P < 0.001$) (Fig. 3*A*) and in muscle, concentric training led to an ~7-fold increase ($P < 0.001$), while eccentric and isometric training induced increases of approximately ~13-fold ($P < 0.001$). However, no significant differences were seen between training types ($P > 0.05$) (Fig. 3*B*).

TGF-β-1 and CTGF

TGF-β-1 and CTGF mRNA were measured with both real-time RT-PCR and Northern blotting, and though fold changes appeared slightly lower with Northern blotting, the observed changes in mRNA levels for TGF-β-1 and CTGF were comparable (Figs 4 and 5). All types of training induced an increase in tendon levels of TGF-β-1 mRNA in a range of 2- to 5-fold ($P < 0.001$), with no difference between training types (Fig. 4*A* and *C*). In muscle, an increase was also seen in TGF-β-1 mRNA in response to all training types ($P < 0.01$); however, eccentric training induced a greater increase than concentric training ($P < 0.05$) (Fig. 4*B* and *D*). For CTGF, no differences were seen between control and trained tendon ($P > 0.05$) (Fig. 5*A* and *C*). In muscle, a moderate increase (1- to 2-fold) in CTGF mRNA was observed in response to eccentric and isometric training ($P > 0.05$), while no change was found after concentric training ($P > 0.05$) (Fig. 5*B* and *D*).

Lysyl oxidase

A marked increased was seen in tendon tissue expression of LOX mRNA in response to all training types (up to 37-fold increase) ($P < 0.001$) (Fig. 6*A*). In muscle, LOX expression increased more moderately (3- to 10-fold) and the effect of concentric training was just below significance

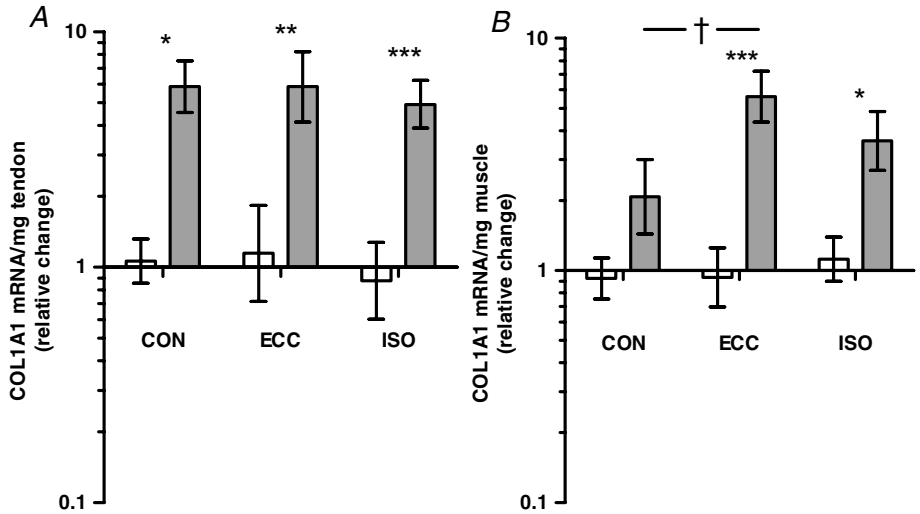


Figure 2. COL1A1 mRNA
COL1A1 mRNA normalized to tissue weight, presented as fold changes relative to the mean of all control values, in Achilles tendon (*A*) and gastrocnemius muscle (*B*) subjected to concentric (CON), eccentric (ECC) and isometric (ISO) training (grey bars) *versus* contralateral controls (white bars). Values are geometric means ± S.E.M. *A*, COL1A1 increased in tendon in response to all training types (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). *B*, muscle COL1A1 mRNA increased in response to ECC and ISO training (*$P < 0.05$, ***$P < 0.001$), and ECC training tended to have greater effect than CON ($^{\dagger}P = 0.1$).

($P = 0.06$) ($P < 0.001$ for ECC and ISO) (Fig. 6*B*). No specific effect was seen of contraction type in either tissue type ($P > 0.05$).

MMPs

MMP-2 mRNA expression appeared to increase moderately in both tendon and muscle in response to all types of training (1- to 3-fold), though not reaching significance for ECC tendon ($P = 0.1$) and CON muscle ($P = 0.09$). No specific effect was seen of contraction mode on MMP-2 expression ($P > 0.05$) (Fig. 6*C* and *D*).

MMP-9 was below detection level in nearly all control samples of both muscle and tendon. In response to training, the number of samples containing detectable levels of MMP-9 mRNA increased significantly ($P < 0.001$) (data not shown). However, even in loaded tissue the measured level of MMP-9 mRNA was still negligible compared with MMP-2 mRNA in both tissue types (data not shown).

TIMPs

Both TIMP-1 and -2 mRNA increased in response to all training types in tendon and muscle tissue ($P < 0.05$) (Fig. 6*E*–*H*). The induction of TIMP-2 was moderate (1- to 4-fold), whereas TIMP-1 was highly induced, especially in muscle tissue (up to 60-fold) (Fig. 6*F*). The effect of eccentric training was greater than concentric on both TIMP-1 and 2 expression in muscle ($P < 0.05$), while in tendon no specific effect was seen of contraction type ($P > 0.05$).

Discussion

The presented findings support the view that tendon and muscle connective tissue adapt to training by increased collagen synthesis and are in agreement with an involvement of transforming growth factor-β-1 (TGF-β-1) in this adaptive response *in vivo*. Furthermore, the results suggest that muscle tissue is more sensitive to differences in contraction type and/or force production than tendon tissue.

In humans, exercise/training has been shown to induce collagen synthesis in both tendon and muscle tissue, as measured by changes in the interstitial pro-collagen I C-terminal peptide (PICP) levels for tendon tissue (Langberg *et al.* 1999, 2001; Hishikawa *et al.* 2001; Heinemeier *et al.* 2003; Crameri *et al.* 2004; Olesen *et al.* 2006*a*), and by increases in incorporation of amino acid tracers for both tendon and muscle (Moore *et al.* 2005; Miller *et al.* 2005). Likewise, earlier animal studies show increased collagen synthesis rates during stretch-induced muscle hypertrophy (Laurent *et al.* 1985). These observations indicate that the supporting connective tissues of the skeletal muscle undergo an adaptive response when subjected to increased loading. In line with this, we find a noticeably increased expression of type I and III collagen in both tendon and muscle in response to short-term strength training in rats. Very few *in vivo* results have been published with regard to tendon expression of collagen mRNA in response to loading. Archambault *et al.* showed increased type III collagen expression in rabbit Achilles tendon in response to 11 weeks of high-frequency loading at low stress/strain

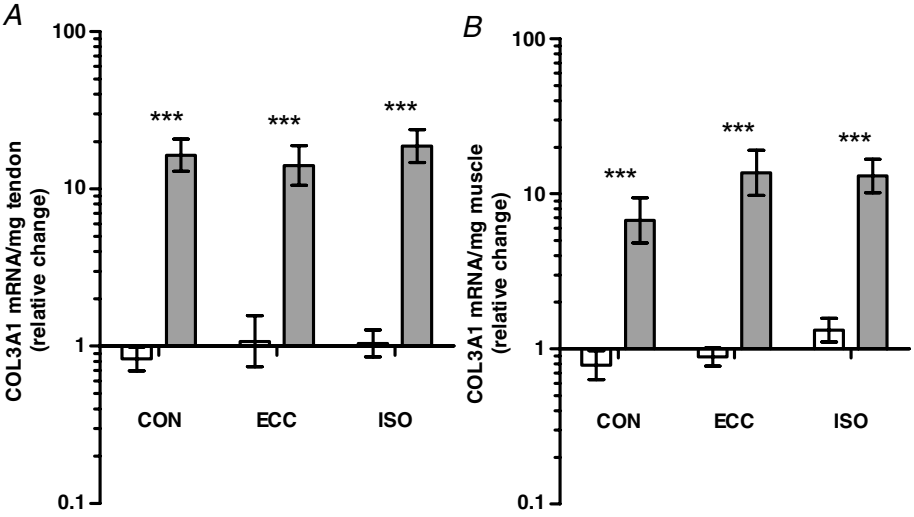


Figure 3. COL3A1 mRNA
COL3A1 mRNA normalized to tissue weight, presented as fold changes relative to the mean of all control values, in Achilles tendon (*A*) and gastrocnemius muscle (*B*) subjected to concentric (CON), eccentric (ECC) and isometric (ISO) training (grey bars) *versus* contralateral controls (white bars). Values are geometric means ± S.E.M. *A* and *B*, COL3A1 increased in both tendon and muscle in response to all training types (***$P < 0.001$), and no difference was seen between training types ($P > 0.05$).

levels, but did not detect any change in type I collagen mRNA levels (Archambault *et al.* 2001). The longer loading duration in that study, compared with the present one, could explain why no change was observed for type I collagen expression, as an increase may have occurred at an earlier time-point. In accordance with the present observations, we have recently shown that functional loading of the plantaris tendon in rats leads to increased expression levels of both type I collagen (after 8 days of loading) and type III collagen (after 2 days of loading) (Olesen *et al.* 2006*b*). Regarding the regulation of collagen expression in skeletal muscle, our findings are in line with two earlier studies showing increases in collagen type I and III mRNA in response to acute strenuous eccentric exercise in both damaged and undamaged rat muscle (Han *et al.* 1999; Koskinen *et al.* 2001), and with findings of increased type I collagen expression in rabbit muscle after long-term jump training (Ducomps *et al.* 2004).

The mechanisms that link mechanical loading during exercise/training to increased expression of collagen in the muscle–tendon unit are still unclear. There are several indications, however, that mechanically induced collagen synthesis in general relies on an increased expression of collagen inducing stress/strain-responsive growth factors, including transforming growth factor-β-1 (TGF-β-1) and connective tissue growth factor (CTGF) (Chiquet *et al.* 2003). A positive correlation between mechanical loading of cells and TGF-β-1 expression has been shown in both *in vitro* and *in vivo* studies, on a large variety of human and animal cell and tissue types (Villarreal & Dillmann,

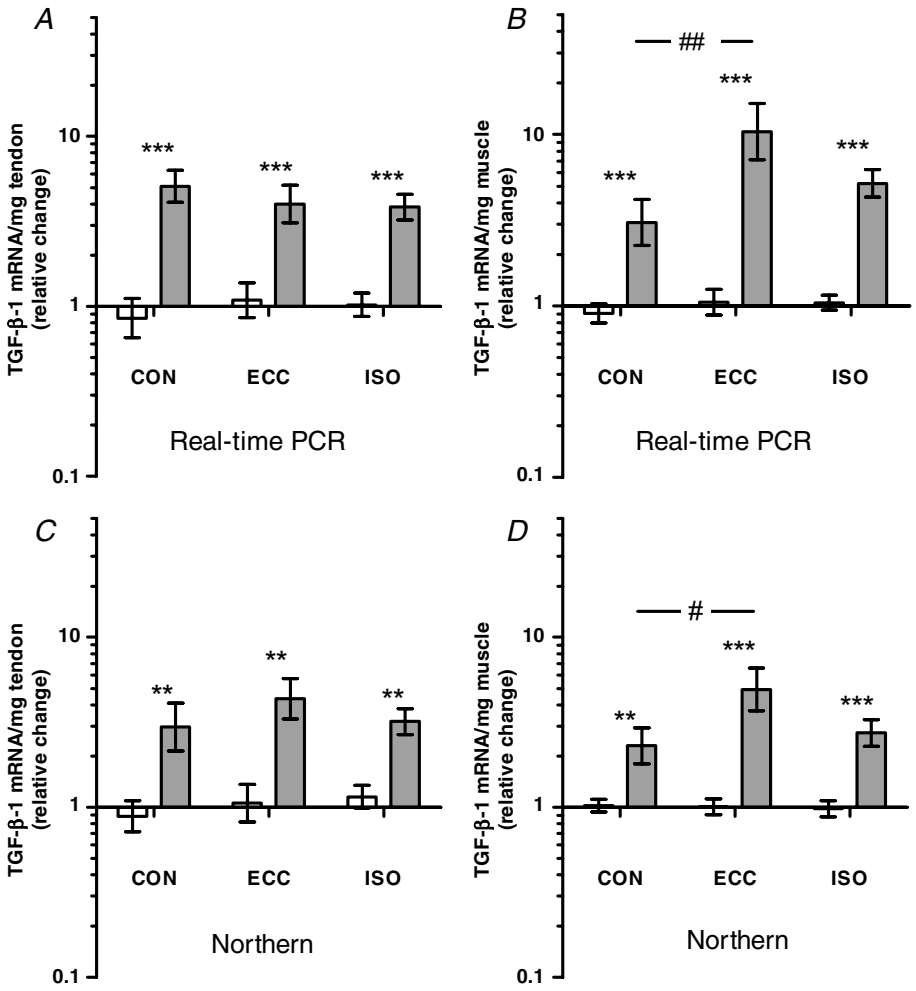


Figure 4. TGF-β-1 mRNA
TGF-β-1 mRNA normalized to tissue weight, presented as fold changes relative to the mean of all control values, in Achilles tendon (*A* and *C*) and gastrocnemius muscle (*B* and *D*) subjected to concentric (CON), eccentric (ECC) and isometric (ISO) training (grey bars) *versus* contralateral controls (white bars). Levels of mRNA were measured with real-time RT-PCR (*A* and *B*) and Northern blotting (*C* and *D*). Values are geometric means ± S.E.M. *A* and *C*, in tendon, TGF-β-1 increased in response to all training types ($^{**}P < 0.01$, $^{***}P < 0.001$). *B* and *D*, in muscle, TGF-β-1 increased in response to all training types ($^{**}P < 0.01$, $^{***}P < 0.001$), but ECC training had greater effect than CON ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

1992; Robbins *et al.* 1997; Cucina *et al.* 1998; Li *et al.* 1998; Gutierrez & Perr, 1999; Lee *et al.* 1999; Cillo *et al.* 2000; Joki *et al.* 2000; O'Callaghan & Williams, 2000; Ruwhof *et al.* 2000; Zheng *et al.* 2001; Skutek *et al.* 2001; Lindahl *et al.* 2002). Importantly, loading-induced type I and/or type III collagen expression appears to depend directly on TGF-β-1 activity in human ligament (Nakatani *et al.* 2002; Kim *et al.* 2002) and patella tendon fibroblast (Yang *et al.* 2004). Thus, the *in vitro* evidence that suggests TGF-β-1 as an important mediator of mechanically induced collagen synthesis in fibroblasts is relatively strong, and the recent findings by Yang *et al.* support such a role for TGF-β-1 in tendon (Yang *et al.* 2004). However, only few *in vivo* data have been published with regard to the role of TGF-β-1 in loaded tendon tissue and while one study supports the idea that TGF-β-1 could be important for Achilles tendon adaptation to uphill running in humans (Heinemeier *et al.* 2003), recent work by Legerlotz *et al.* showed no change in TGF-β-1 expression in response to different types of long-term training in rats (Legerlotz *et al.* 2007). However, in that study mRNA levels were only measured after 12 weeks of training and an increase in TGF-β-1 expression may well have been present at earlier stages. The present *in vivo* observations of increased TGF-β-1 mRNA levels in the early phase of resistance training, combined with an elevated type I and III collagen expression, provide significant support for the suggestion of TGF-β-1 as a link between exercise/training and collagen expression in tendon tissue.

In skeletal muscle, TGF-β-1 has been suggested to be involved in both exercise-induced angiogenesis (Breen *et al.* 1996; Gavin & Wagner, 2001) and pathological

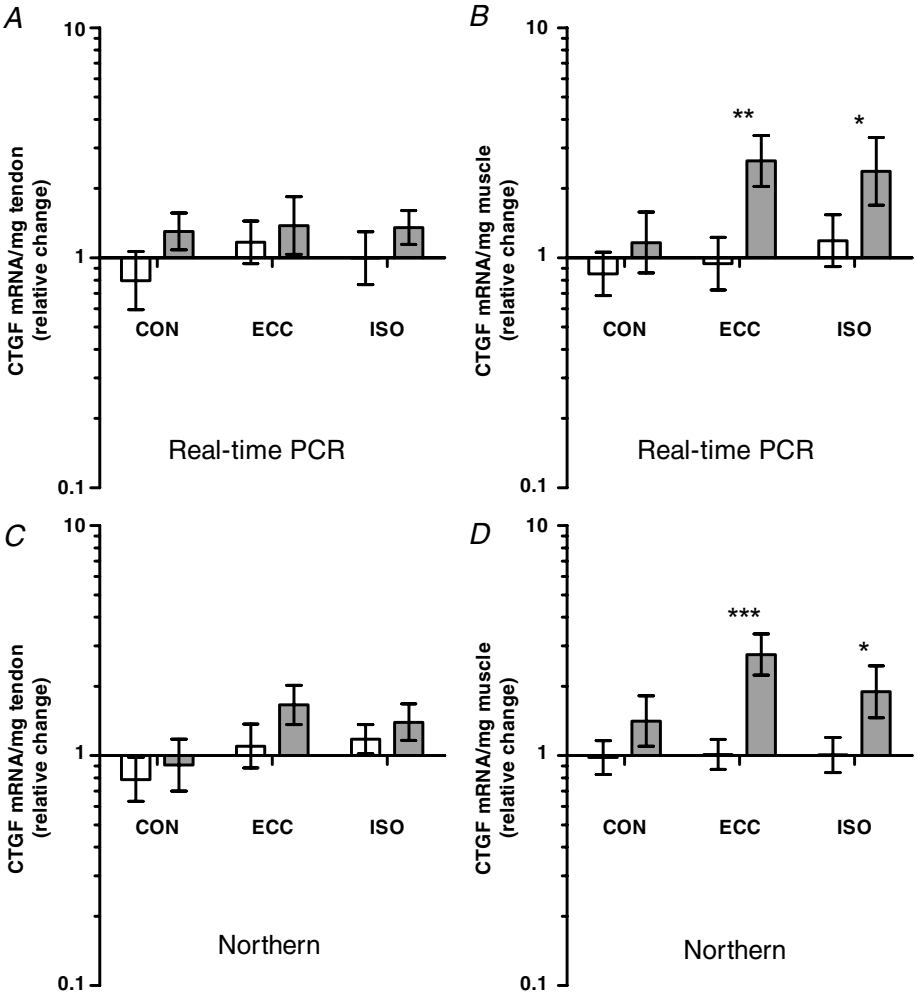


Figure 5. CTGF mRNA
CTGF mRNA normalized to tissue weight, presented as fold changes relative to the mean of all control values, in Achilles tendon (*A* and *C*) and gastrocnemius muscle (*B* and *D*) subjected to concentric (CON), eccentric (ECC) and isometric (ISO) training (grey bars) *versus* contralateral controls (white bars). Levels of mRNA were measured with real-time RT-PCR (*A* and *B*) and Northern blotting (*C* and *D*). Values are geometric means ± S.E.M. *A* and *C*, in tendon, no changes were seen in CTGF mRNA levels ($P > 0.05$). *B* and *D*, in muscle, CTGF increased in response to ECC and ISO (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), while no effect was seen of CON ($P > 0.05$).

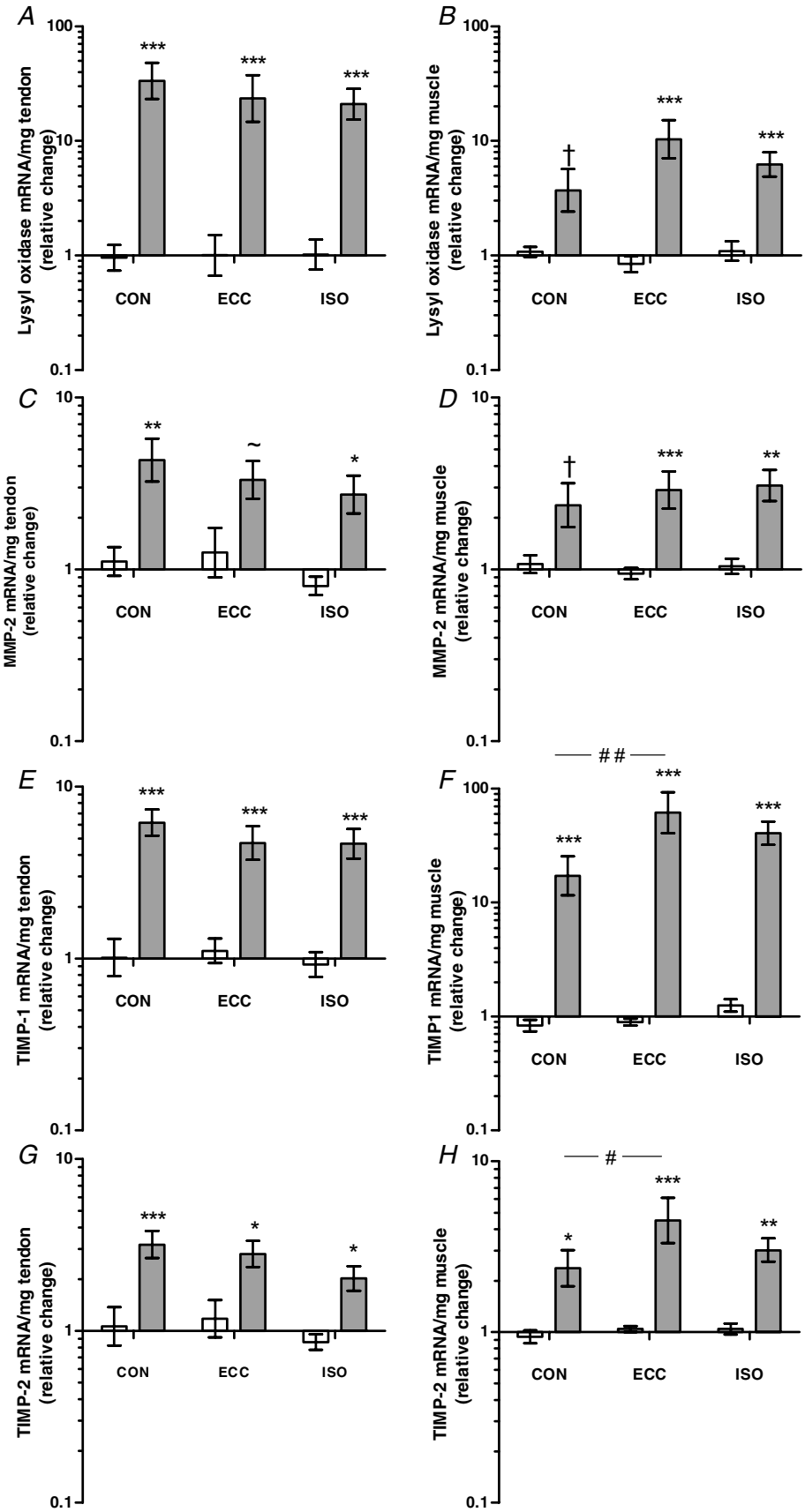
muscle fibrosis (Li *et al.* 2004), and increases in TGF-β-1 mRNA have been observed in response to muscle damaging eccentric exercise in humans (Hamada *et al.* 2005) and to acute endurance exercise and short-term endurance training in rats (Breen *et al.* 1996; Gavin & Wagner, 2001). In the present study we find an elevated expression of TGF-β-1 in skeletal muscle, along with elevated collagen mRNA levels, in response to short-term training. These findings, combined with the well-known collagen-inducing effect of TGF-β-1, support a role for this growth factor in relation to training-induced non-pathological muscle ECM production. The present and earlier studies do not specify the source of TGF-β-1 mRNA, and though myotubes do not appear to increase TGF-β-1 expression in response to strain (Tsivitse *et al.* 2005), indicating that other cell types present in skeletal muscle (e.g. fibroblasts, vascular cells) are a likely source, further investigation is needed in order to determine which cell types are responsible for the production of TGF-β-1 mRNA. This could help to pinpoint the functions of TGF-β-1 in skeletal muscle. Importantly, however, it should be considered that a general increase in TGF-β-1 protein concentration, regardless of the source, is likely to affect gene expression in a number of cell types, including collagen expression in muscle fibroblasts.

Connective tissue growth factor (CTGF) – a potent stimulator of collagen synthesis – is induced by mechanical stimuli (Schild & Trueb, 2002) and appears to be involved in mechanically induced collagen synthesis in certain cell types (Hishikawa *et al.* 2001). In fibroblasts, CTGF is induced by TGF-β-1 and, by acting as a down-stream mediator, CTGF appears to be at least partly responsible for the collagen-inducing actions of TGF-β-1 (Grotendorst *et al.* 1996; Duncan *et al.* 1999). The role of CTGF in tendon and muscle is largely unknown. However, one recent study by Nakama *et al.* has shown an increase in the amount of CTGF-positive (immuno-stained) cells in rabbit tendon after 80 accumulated hours of low force repetitive loading (Nakama *et al.* 2006). In contrast, we did not observe any significant change in tendon CTGF mRNA levels in response to training. However, the results of the present study are not easily compared with the findings of Nakama *et al.* as the amount and duration of stress/strain applied to tendons in the two studies are fundamentally different. Furthermore, the loading regime applied by Nakama *et al.* led to microtears in the tendon tissue and initiation of a wound-healing response might explain the observed increase in CTGF staining (Nakama *et al.* 2005). Our findings do not indicate an involvement of CTGF in the adaptation of tendon tissue to training; however, an increase in CTGF expression may have occurred at an earlier time-point than the one chosen for measurement, and further experiments are needed to clarify the role of CTGF in tendon.

The function of CTGF in skeletal muscle is unknown; however, recent observations show that rat myoblasts and myotubes express CTGF *in vitro*, and that its expression is increased by TGF-β-1 (Obreo *et al.* 2004; Maeda *et al.* 2005). Furthermore, several types of fibroblast increase their expression of CTGF in response to both mechanical loading (Schild & Trueb, 2002) and exogenous TGF-β-1 (Grotendorst *et al.* 1996; Duncan *et al.* 1999; Blalock *et al.* 2003) and a similar response in muscle ECM fibroblasts seems likely. Thus, an increased TGF-β-1 expression, along with the mechanical stimulus during training, are possible explanations for the observed increases in muscle CTGF expression in response to eccentric and isometric training. A possible connection between TGF-β-1 and CTGF expression is supported by the fact that concentric training had a relatively low effect on TGF-β-1 expression and no effect on CTGF expression.

In addition to increased collagen synthesis, the adaptation of tendon and muscle connective tissue to training is thought to involve changes in the quality of the tissue structure, such as increased levels of cross-linking between collagen molecules (Kovanen *et al.* 1984; Buchanan & Marsh, 2002; Kubo *et al.* 2002). The enzyme lysyl oxidase (LOX) could be important for this type of adaptation as it facilitates the formation of lysine-derived covalent cross-links between and within collagen molecules, leading to stabilization and strengthening of fibrillar collagen structures (Kagan & Li, 2003). Though one recent study indicates that habitual tendon loading may be necessary to maintain normal LOX expression (Arruda *et al.* 2007), the present study is apparently the first to show that LOX mRNA expression in tendon is highly responsive to mechanical stimuli. A similar trend was seen in muscle, which is in accordance with earlier work by Han *et al.* (1999), and although these observations should be supplemented with more mechanistic experiments (and verified on the protein level) our findings definitely support the idea of an increased collagen cross-linking as part of tendon and muscle connective tissue adaptation to loading.

A transient increase in collagen degradation appears to be an additional aspect of the tendon-tissue response to mechanical loading (Langberg *et al.* 2001; Olesen *et al.* 2006*a*), and matrix metalloproteinases, MMP-2 and -9, which can degrade intact fibrillar collagen, may be involved in this process (Visse & Nagase, 2003). We have previously shown a pronounced increase in MMP-9 gelatinolytic activity in dialysate obtained from human peritendinous tissue immediately after acute exercise (Koskinen *et al.* 2004), and recent data suggest increased levels of both MMP-9 protein and mRNA in skeletal muscle shortly after a single exercise bout in men (Rullman *et al.* 2007). In the present study, however, MMP-9 mRNA was below the detection level in non-loaded muscle/tendon tissue and barely detectable in loaded tissue. In support of



this, Koskinen *et al.* could not detect MMP-9 mRNA in control or eccentrically loaded rat muscle (Koskinen *et al.* 2001), and similar observations have recently been made in rat muscle subjected to long-term high-intensity exercise (Carmeli *et al.* 2005). Additionally, studies on human cadaver tissue suggest very low MMP-9 expression in tendon (Riley *et al.* 2002). This discrepancy could have several explanations, one being that the two studies, which show a pronounced loading-induced increase in MMP-9 activity, both included traumatic interventions prior to exercise (i.e. insertion of microdialysis probes or multiple muscle biopsy procedures) (Koskinen *et al.* 2004; Rullman *et al.* 2007), which may have contributed to the post-exercise increase in MMP-9 activity/expression (Madlener, 1998). However, this is speculative, and evidently further studies are needed to clarify the presence and importance of MMP-9 in tendon and skeletal muscle.

Though MMP-2 is known to be expressed in both human and rat tendon tissue (Riley, 2005; Jones *et al.* 2006; Legerlotz *et al.* 2007), the relationship between mechanical loading and MMP-2 expression in tendon is unclear. In the present study we found a moderate increase in MMP-2 expression in loaded tendon, and previously we have observed a transient decrease followed by an increase in MMP-2 gelatinolytic activity in human Achilles peritendon tissue after acute exercise (Koskinen *et al.* 2004). Meanwhile, a recent study by Legerlotz *et al.* did not indicate any regulation of tendon MMP-2 mRNA expression in response to long-term strength training in rats (Legerlotz *et al.* 2007). Our results on skeletal muscle are in line with earlier studies showing elevated MMP-2 expression in rat muscle after acute downhill running (Koskinen *et al.* 2001) and after long-term endurance training (Carmeli *et al.* 2005). Considering that MMP-2 alone can mediate degradation of mature collagen molecules (Visse & Nagase, 2003), our findings could support a loading-induced collagen degradation. However, along with the increased MMP-2 expression, we observed a significant induction of the tissue inhibitors of metalloproteinases (TIMPs), especially TIMP-1 (Fig. 6), and given their inhibitory effect on MMP activity it is not possible to predict the net effect on collagen turnover.

With regard to the specific effects of different contraction types, we saw a similar increase in tendon expression of collagens and regulatory factors in response to the three different loading types. In muscle, however, eccentric training had a greater effect than concentric training on expression on TGF-β-1 and TIMPs. Furthermore, concentric training did not change muscle CTGF expression, while an effect was seen of both eccentric and isometric training. Taking into account the well-known effect of both TGF-β-1 and CTGF on collagen expression, it could be expected that eccentric training would have a greater effect on muscle collagen expression than concentric. In support of this, collagen I expression was not significantly induced by concentric training, while a 4-fold increase was seen after eccentric loading, and although the difference between eccentric and concentric loading did not reach significance ($P = 0.1$), we cannot conclude that collagen expression in muscle is unrelated to specific training types. The differences seen in the muscle response to specific contraction types could possibly be linked to differences in generation of shear stress between muscle fibres and ECM, as this could lead to a differential mechanical loading on both muscle fibres and connective tissue cells. However, the differences in force production between the training modes are also a likely explanation. The adaptive response of muscle was generally lowest after concentric training and highest in response to eccentric training, which corresponds well with forces generated during the different contraction types (Fig. 1). The possibility that the muscle response is primarily related to the force production is supported by recent data showing an equal increase in the expression of several anabolic genes, as well as type III collagen, in rat skeletal muscle in response to concentric, eccentric and isometric training with equal force–time integrals (Garma *et al.* 2007). Although the differential muscle response may simply relate to force production, it is still interesting that skeletal muscle tissue appears sensitive to the specific force development (and/or contraction type), while no differential response is seen in tendon, even though the stress/strain experienced by this tissue is directly related to the force generated by muscle. These observations, combined with our corresponding results on

Figure 6. Lysyl oxidase, MMP-2, TIMP-1 and TIMP-2 mRNA
Lysyl oxidase, MMP-2, TIMP-1 and TIMP-2 mRNA normalized to tissue weight, presented as fold changes relative to the mean of all control values, in Achilles tendon (*A*, *C*, *E* and *G*) and gastrocnemius muscle (*B*, *D*, *F* and *H*) subjected to concentric (CON), eccentric (ECC) and isometric (ISO) training (grey bars) *versus* contralateral controls (white bars). Values are geometric means ± S.E.M. *A* and *B*, LOX mRNA levels increased in tendon and muscle in response to all training types, though this only tended to be significant for CON trained muscle (†$P = 0.06$, ***$P < 0.001$). *C*, tendon MMP-2 mRNA increased after CON and ISO and tended to do so after ECC (†$P = 0.1$, *$P < 0.05$ **$P < 0.01$). *D*, ECC and ISO (and possibly CON) loading-induced MMP-2 mRNA expression in muscle (†$P = 0.09$, **$P < 0.01$, ***$P < 0.001$). *E–H*, TIMP-1 and TIMP-2 mRNA levels were increased in both tendon and muscle in response to all training types ($P < 0.05$) and in muscle tissue the effect of eccentric training was greater than concentric ($P < 0.05$).

anabolic growth factors (Heinemeier *et al.* 2007), indicate a more specific adaptive response in muscle, compared with tendon, and potentially, certain training modes may lead to an unbalanced adaptation of tendon *versus* muscle.

Eccentric training might have been expected to induce a specific tendon response, as several studies in humans show a superior effect of eccentric loading in treatment of achilles tendinosis compared with other loading types (e.g. Mafi *et al.* 2001; Silbernagel *et al.* 2001). Thus, based on the present observations, it could be speculated that the beneficial effect of eccentric training in the treatment of tendon pathology is unrelated to collagen and growth factor expression. However, when considering the differences in species, duration of training intervention and range of movement during contraction, this is by no means certain.

In conclusion, we have demonstrated that short-term training induces collagen expression in both skeletal muscle and tendon tissue, and our findings support the hypothesis that TGF-β-1 acts as a mediator of training-induced collagen expression in these tissues. Furthermore, eccentric training appears to have a larger potential than concentric training for increasing the expression of collagen-inducing growth factors in muscle. This may be connected to a greater force production during eccentric contraction, or perhaps to a greater local shear stress in the muscle tissue. The changes seen in tendon were not dependent on stress/strain levels or contraction type, which indicates that this tissue is less sensitive than skeletal muscle to differences in mechanical stimulus.

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